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Minor Histocompatibility Antigen-Specific Cytotoxic T Lymphocytes Generated with Dendritic Cells from DLA-Identical Littermates

George E. Georges,[1,2] *Marina Lesnikova,*[1] *Rainer Storb,*[1,2] *Murad Yunusov,*[1] *Marie-Térèse Little,*[1] *Richard A. Nash*[1,2]

[1]Clinical Research Division, Fred Hutchinson Cancer Research Center, and [2]Department of Medicine, University of Washington, Seattle, Washington

Correspondence and reprint requests: George E. Georges, MD, Fred Hutchinson Cancer Research Center, 1100 Fairview Ave. N., D1-100, P.O. Box 19024, Seattle, WA 98109-1024 (e-mail: ggeorges@fhcrc.org).

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ABSTRACT

Donor cytotoxic T lymphocytes (CTL) specific for minor histocompatibility antigens (mHA) mediate the graft-versus-host effect whereas host mHA-specific CTL mediate graft rejection in the setting of major histocompatibility complex identical allogeneic hematopoietic stem cell transplantation. Development of a large animal model from which mHA-specific CTL can be isolated would accelerate translation in clinical studies to improve control of the graft-versus-host effect as well as prevention of graft rejection in sensitized hosts. The aims of the current study were to isolate mHA-specific CTL from dog leukocyte antigen–identical littermate nonsensitized recipients before transplantation, from stable mixed hematopoietic chimeras, and from dogs sensitized to mHA after graft rejection. Donor dendritic cells (DCs) were cultured from bone marrow–derived $CD34^+$ cells and were used to stimulate recipient T lymphocytes on days 1, 10, and 20 of CTL culture. We reliably generated and expanded mHA-specific CTL ex vivo from sensitized dogs that were given a donor-specific blood transfusion to boost immune recall after graft rejection after a nonmyeloablative transplantation. The mHA-specific cytotoxicity measured by ^{51}Cr release assay was enriched from less than 5% in the starting population of sensitized peripheral blood mononuclear cells to a median of 63% after 4 weeks in CTL culture. The expanded mHA-specific CTLs were not tissue-specific: hematopoietic cells, fibroblast, and stromal cell lines were lysed in an mHA-specific manner. Allogeneic DCs, but not peripheral blood mononuclear cells, were necessary for stimulating ex vivo expansion of mHA-specific CTL. We were unable to generate mHA-specific CTL from nonsensitized dogs before transplantation, from previously sensitized dogs but without recent recall immunization, or from stable mixed hematopoietic chimeras. We conclude that after recent in vivo sensitization, large-scale ex vivo expansion of mHA-specific CTL was feasible using allogeneic DCs.

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KEY WORDS

Minor histocompatibility antigen • Cytotoxic T lymphocyte • Graft rejection • Dendritic cell • Nonmyeloablative allogeneic hematopoietic stem cell transplantation

INTRODUCTION

After major histocompatibility complex (MHC)–identical hematopoietic stem cell transplantation (HSCT), donor-derived, minor histocompatibility antigen (mHA)–specific cytotoxic T lymphocytes (CTL) mediate graft-versus-host disease (GVHD) and the graft-versus-leukemia (GVL) effect whereas host-derived mHA-specific CTLs mediate graft rejection [1]. In the nonmyeloablative HSCT setting, the role of mHA-specific CTLs is particularly important because the intensity of the conditioning regimen is significantly reduced and postgrafting pharmacological immunosuppression is intensified to control both GVHD and graft rejection [2,3]. In the canine HSCT model, donor T cells, including mHA-specific CTLs, are necessary for sustained engraftment [4] and conversion to complete donor chimerism [5]. Likewise, we hypothesized that host-derived mHA-specific CTL mediated graft rejection if the host was sensitized previously to donor antigens [6] or if the condi-

tioning regimen or postgrafting immunosuppression was insufficient [2,7-9]. Development of a large animal model for studying mHA-specific CTLs could accelerate translation in clinical studies to further decrease the intensity of conditioning regimen while enhancing the GVL effect and preventing GVHD or preventing graft rejection in sensitized hosts.

Previously, it was difficult to reliably generate canine mHA-specific T cells ex vivo, and expansion of CTLs was very limited [10]. We used the recently isolated and characterized canine dendritic cells (DCs) [11] to stimulate the ex vivo expansion of mHA-specific CTL from dog leukocyte antigen (DLA)–identical littermates. We asked if mHA-specific CTLs could be isolated from healthy dogs before transplantation, from stable mixed hematopoietic chimeras, and from dogs sensitized to mHAs after graft rejection.

MATERIALS AND METHODS

Experimental Animals

Litters of random-bred dogs weighing from 8.0 to 14.7 kg and 6 to 12 months old were raised at the Fred Hutchinson Cancer Research Center or obtained from commercial kennels licensed by the United States Department of Agriculture. Research was conducted per the principles in the *Guide for Laboratory Animal Facilities and Care* (National Academy of Sciences, National Research Council). The Institutional Animal Care and Use Committee of the Fred Hutchinson Cancer Research Center approved the research protocol. Kennels are certified with the American Association for Accreditation of Laboratory Animal Care. During the study, dogs were examined at least twice daily. DLA-identical littermate pairs were chosen on the basis of identical highly polymorphic MHC class I and class II microsatellite markers [12]. Specific DLA DRB1 allelic identity was determined using direct sequencing [13]. Refer to Table 1 for summary of the experimental dogs used in this study.

Sensitization to mHAs

Treatment Group A. Pretransplantation sensitization to mHAs was performed in 4 bone marrow recipient dogs. Each dog was given 3 serial nonirradiated whole blood (heparinized) transfusions (50 mL each) infused on days −24, −17, and −10 from the respective DLA-identical littermate donor. On day 0, sensitized recipients were given 2 Gy of total body irradiation (TBI) at 0.07 Gy/min from a high-energy linear accelerator source (Varian CLINAC 4, Palo Alto, CA) followed by transplantation of bone marrow [14] from the DLA-identical littermate. Dogs received combined mycophenolate mofetil (MMF), 10 mg/kg subcutaneously, twice daily, and cyclosporine (CSP), 15 mg/kg orally, twice daily, for 4 and 5 weeks after transplantation, respectively. All 4 bone marrow recipients were shown to be sensitized to donor mHAs with immediate rejection of their donor grafts with complete reconstitution of autologous hematopoiesis, confirmed using microsatellite marker studies [15].

Six to 8 months after marrow transplantation, the 4 previously sensitized dogs received a transfusion of 50 mL of nonirradiated whole blood from the rejected DLA-identical marrow donor to boost the immune recall to mHAs. Ten days later, 4×10^7 of peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Hypaque (1.074 specific gravity) density gradient centrifugation of 40 to 60 mL of heparinized blood from the mHA-sensitized dogs. Treatment group A is hereafter also referred to as previously sensitized and given recall immunization.

To determine if recall immunization or prior in vivo

Table 1. *Experimental Treatment Groups* and DLA-Identical Littermate Pairs for Isolation of mHA-Specific CTLs*

Treatment Group A	Treatment Group B	Treatment Group C	Treatment Group D
• Sensitized to mHA with donor-specific whole blood transfusion • Confirmed sensitization to donor mHA owing to graft rejection after nonmyeloablative HSCT in prior 6-8 mo • Day −10 recall immunization	• Previously sensitized to donor mHA owing to graft rejection after nonmyeloablative HSCT in prior 6-8 mo • No recall immunization	• Nonsensitized PBMCs obtained before scheduled transplantation	• Stable mixed hematopoietic chimera 8-22 mo after nonmyeloablative HSCT
Donor → recipient: DLA-identical littermate pairs for nonmyeloablative HSCT			
E614 → E608 E768 → E765 E892 → E894 E903 → E902	E699 → E692 G006 → G005	E212 → E214 E991 → E988	E874 → E873 E942 → E941 E972 → E970 E991 → E988

All treatment groups: PBMC for mHA-specific CTL culture obtained from the designated nonmyeloablative HSCT recipient dog after the indicated treatment.

sensitization to mHAs was necessary for generating mHA-specific CTL, 2 control groups were studied.

Treatment Group B. PBMCs were obtained from 2 dogs, G005 and E692, that 6 and 8 months previously, respectively, had rejected a DLA-identical littermate bone marrow graft after 1 Gy of TBI conditioning. G005 had received CSP/MMF and E692 received CSP only as posttransplantation immunosuppression. PBMCs for in vitro studies were obtained from these dogs without preceding recall immunization with donor-specific blood transfusion. Because of the prior graft rejection, these dogs were sensitized previously to donor mHAs, similar to group A. They served as a control for group A to determine if boosting immune recall to mHAs after graft rejection was necessary for ex vivo generation of mHA-specific CTL. Group B also is referred to as previously sensitized without recall immunization.

Treatment Group C. PBMC were obtained from 2 dogs before bone marrow transplantation that were not sensitized to their respective DLA-identical littermate. For each dog pair studied, 1 to 3 independent experiments to generate mHA-specific CTLs ex vivo were performed.

Establishment of Mixed Hematopoietic Chimerism

Treatment Group D. Stable mixed donor/host hematopoietic chimerism was established in 4 dogs with a nonmyeloablative regimen of TBI, DLA-identical marrow, and a brief course of postgrafting CSP/MMF immunosuppression as previously described [2,16]. The bone marrow recipients, E873, E941, E970, and E988, had 15%, 55%, 80%, and 40% stable donor chimerism in the PBMC fraction, respectively, which remained unchanged during the 20-week period before obtaining blood for the mHA-CTL studies. PBMCs obtained from these 4 mixed chimeras at 8 to 22 months after transplantation were evaluated for the ability to generate mHA-specific CTLs against DCs obtained from the respective DLA-identical littermate bone marrow donor.

Generation of DCs

DCs from the respective bone marrow donor were used to stimulate and expand mHA-specific CTLs cultured from the sensitized (treatment group A and B), nonsensitized (treatment group C), or mixed chimeric (treatment group D) dog PBMCs. DCs were cultured from bone marrow $CD34^+$ cells with modification of the previously described method [11]. Briefly, bone marrow was aspirated from the femora of anesthetized dogs [14], hemolyzed, and the pelleted cells were resuspended in Hank's balanced salt solution with 2% heat-inactivated horse serum at 1×10^8/mL. Cells were incubated with canine anti-CD34 monoclonal antibody 2E9 [17] 40 μg/mL for 30 minutes at 4° C and washed. After incubation with 100 μL/mL IgG1-isotype-specific immunomagnetic beads (Miltenyi Biotech, Auburn, CA) for 20 minutes, cells were loaded onto an AutoMACS cell separation device (Miltenyi Biotech) according to the manufacturer's instructions for positive cell selection. An aliquot of the adsorbed cell fraction was reserved for flow cytometry analysis to determine $CD34^+$ cell purity [18]. The $CD34^+$ enriched cells were seeded at 0.5×10^6/well in 24 well plates (Costar, Corning, NY) for 14 days in Iscove's medium containing 10% autologous heat inactivated dog serum, 5 ng/mL granulocyte macrophage colony-stimulating factor (Immunex, Seattle, WA), 200 ng/mL flt3 ligand, and 10 ng/mL tumor necrosis factor–α (TNFα) (R&D Systems, Minneapolis, MN). The medium was changed every other day beginning on day 5 of culture, and 1 to 1.5 mL of fresh medium with cytokines was added. The cells were split 1:2 after 7 days in culture. The DCs were washed and irradiated (22 Gy, ^{137}Cs source) before use as stimulator cells.

Ex Vivo Expansion of mHA-Specific CTLs

Responder PBMCs were cultured (2×10^6/well, Costar) with the respective DLA-identical littermate DCs on day 1 and restimulated with DCs on day 10 and 20 of culture at a 10:1 ratio, respectively. Interleukin-2 (10 IU/mL, Chiron, Emeryville, CA) was added on days 12 and 22. When PBMCs instead of DCs were used as irradiated stimulators, culture conditions were the same except the responder to stimulator ratio was 1:1. Cells were grown in Iscove's medium containing 10% heat inactivated serum of the dog from which the stimulator DCs (or PBMCs) were obtained, 2 mmol/L of L-glutamine, 1 mmol/L of nonessential amino acids, 1 mmol/L of sodium pyruvate, 50 IU/mL of penicillin, and 50 mg/mL of streptomycin. Before each restimulation, mHA-specific CTL activity was assessed using the following: (1) proliferation assay (^{3}[H]TdR incorporation), (2) specific cytotoxicity assay (^{51}chromium release), (3) immunophenotyping studies, and (4) measurement of cell expansion (cell count with trypan blue dye exclusion).

Cell Proliferation Assay

In 6 to 12 replicate wells in 96-microwell U-bottom tissue culture plates (Costar), 1×10^5 responder PBMCs were cultured with γ-irradiated (22 Gy, ^{137}Cs source) stimulator cells, either DCs (2×10^4/well) or PBMCs (1×10^5/well). Responder cells for secondary stimulation were obtained from bulk cultures at day 10 after primary stimulation without the subsequent addition of interleukin-2. Cells were pulsed for 18 hours with tritiated thymidine (^{3}[H]TdR, 0.037 mBq/well, Amersham, St. Louis, MO) at 4, 5, and 6 days after primary stimulation or at 3, 4, and 5 days after secondary stimulation, and then harvested (Packard, Meridien, CT). Counts per

minute (CPM) were measured using β-scintillation counter (Packard).

Analysis of Cytotoxicity

Cytotoxicity of mHA-specific T cells was measured using a standard ^{51}Cr release assay. Briefly, 1×10^6 target cells, either 1×10^6 concanavalin-A stimulated dog PBMCs (con-A blasts) or $CD34^+$-derived DCs, were incubated with 1.85 mBq ^{51}Cr (Dupont, Boston, MA) for 1 hour at 37°C. The target cells were washed 5 times and then incubated for 4 hours with effector CTLs at various effector to target ratios in triplicate at 200 μL/well. The percentage of specific lysis was calculated according to the standard formula [19]. Mean values are based on 3 to 4 replicates ± 95% confidence interval.

Alloreactivity to Bone Marrow Stroma and Fibroblasts

In preparation for assessing mHA-specific reactivity to stroma and fibroblasts, stromal cells were obtained using marrow aspirate and placed in long-term marrow culture with RPMI, 12.5% horse serum, 12.5% dog serum, 10^{-6} mol/L hydrocortisone, 0.1% β-mercaptoethanol, plus the supplements used in CTL medium. Bone marrow-derived fibroblasts were cultured in Iscove's medium containing 10% fetal bovine serum for 5 to 6 weeks before replating for the cytotoxicity assay [20]. The mHA-specific CTLs cultured for 28 days from sensitized dogs with recall immunization (treatment group A) were added (1×10^5, 0.5×10^5, 0.25×10^5 cells/well) to a 96-well flat-bottom plate (Costar) containing either confluent DLA-identical littermate, autologous, or third party stromal cells or fibroblasts previously pulsed for 1 hour with 0.037 mBq ^{51}Cr per well and extensively washed. After 4 hours, culture supernatants were harvested, CPM were measured using γ-counter, and percentage of cytotoxicity was calculated using the standard formula: ([experimental CPM]-[spontaneous CPM]) ÷ ([maximum CPM]-[spontaneous CPM]) × 100. Significant lysis of stroma or fibroblasts was defined as 1.5 × control lysis [21]. Five to 10 replicate wells per assay were performed.

Monoclonal Antibodies

The following canine monoclonal antibodies (MoAbs) specific for cell surface antigens were used: CD34 (2E9, immunoglobulin [Ig]G1) [18], HLA-DR (cH81.98, IgG2a, provided by Michel Pierres, Marseilles, France) [22], and CD14 (Tuk4, IgG2a, Dako, Carpenteria, CA). In addition, CD3ε (CA17.6F9, IgG2b), CD4 (CA13.1E4, IgG1), CD8 (CA9.JD3, IgG2a), CD1c (CA13.9H11, IgG1), and CD11c (CA11.6A1, IgG1) canine-specific MoAbs were provided by Peter Moore, University of California Davis [5,11]. Biotinylated and fluorescein isothiocyanate–labeled negative murine isotype control MoAbs were purchased from Dako.

Flow Cytometric Analysis of Cells

Freshly isolated and cultured cells were analyzed using flow cytometry for expression of cell surface antigens using the fluorescein isothiocyanate–conjugated or biotinylated MoAbs described above at 4 μg/mL. Cell incubations and washes were completed at 4°C in Hank's balanced salt solution with 2% heat-inactivated horse serum followed by secondary staining with streptavidin-phycoerythrin. Propidium iodide (1 μg/mL) was added to exclude dead cells. Analysis was performed on a FACScalibur flow cytometer (Beckton Dickinson, San Jose, CA) with WinList software (Verity Software House, Topsham, ME).

RESULTS

DCs

In the experiments using DCs, the initial $CD34^+$ purity ranged from 46% to 89% (median, 55%). After 14 days of culture in DC medium, >80% of cells had the classic DC veiled morphology. The DC phenotype was confirmed using flow cytometry with >90% MHC class II bright expression, 85% to 99.5% CD14-, >90% $CD1c^+$, and 32% to 55% $CD11c^+$ expression. Cultured DCs previously were shown to have 23- to 40-fold increased allostimulatory activity compared with PBMCs in primary mixed lymphocyte reaction assays in the MHC-mismatched setting [11].

DCs versus PBMCs as Stimulators of mHA-Specific CTLs

We compared DCs with PBMCs as irradiated stimulator cells for generating mHA-specific CTLs from dogs previously sensitized and given recall immunization (treatment group A). Figure 1 shows DCs were superior to PBMCs as stimulators for generating mHA-specific proliferation after primary and secondary stimulation. The difference between DC and PBMC allostimulatory activity was most apparent after secondary stimulation when DCs generated a 13-fold increase in proliferation over PBMC at day 14 in culture.

mHA-Specific CTLs Isolated from Sensitized Dogs

Donor DCs were added as stimulator cells to recipient PBMCs on days 1, 10, and 20 of culture. After 28 days in culture, CTLs derived from dogs previously sensitized and given recall immunization (treatment group A) showed a high level of mHA-specific lysis. After the third DC stimulation, there was 32% to 78% (median, 63%) specific CTL lysis of the respective DLA-identical littermate DC targets. There was no significant difference in assessment of mHA-specific

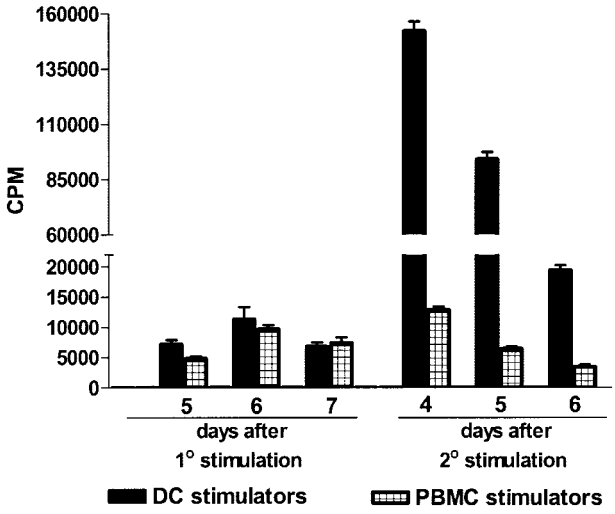

Figure 1. Proliferation of mHA-specific CTLs was enhanced with allogeneic DCs as stimulators. Representative results (n = 6) from a single dog pair are shown. In 6 to 12 replicate wells in 96-well plates, 1×10^5 responder PBMCs from dog E608 (treatment group A, previously sensitized and given recall immunization from dog E614) were cultured with irradiated E614 stimulator cells, either DCs (solid bars, 2×10^4/well) or PBMCs (thatched bars, 1×10^5/well). Cells were pulsed for 18 hours with ^{3}[H]TdR at 4, 5, and 6 days after primary stimulation, or at 3, 4, and 5 days after secondary stimulation, and then harvested. Results are shown as CPM ± standard error.

cytotoxicity with DCs as targets (Figure 2A) compared with con A blasts (Figure 2B). Freshly isolated PBMCs from these sensitized dogs had insignificant lysis of targets (<5%). Control experiments (Figure 2C) showed the mHA-specific CTLs had consistently insignificant lysis of autologous target cells and a panel of 3 to 4 DLA-nonidentical, unrelated third party target cells. This confirmed the observation that the cytolytic activity was specific for mHAs. The mHA-specific CTL activity measured using the percentage of specific lysis was enriched after successive DC stimulation of sensitized PBMCs (Figure 2D). There was insignificant enrichment of mHA-specific CTL activity when DLA-identical PBMCs were used as stimulators (Figure 2E). This showed that DCs were necessary for ex vivo expansion of mHA-specific CTLs.

Requirement for In Vivo Sensitization and Recall Immunization to mHA

In vivo sensitization and recent recall immunization to mHA was necessary for efficiently generating mHA-specific CTLs ex vivo. PBMCs from nonsensitized dogs (treatment group C) cultured under the same conditions used for expansion of mHA-specific CTLs from sensitized dogs did not effectively lyse the respective DLA-identical littermate DCs or con A blasts. In 4 independent experiments, cells from nonsensitized dogs assayed after third DC stimulation had ≤15% mHA-specific lysis at a 20:1 effector to target ratio (Figure 2F).

We asked whether boosting immune recall to mHAs was necessary for ex vivo expansion of mHA-specific CTLs from previously sensitized dogs. PBMCs were obtained from 2 dogs (treatment group B) that 6 to 8 months previously had rejected DLA-identical bone marrow grafts after a nonmyeloablative conditioning regimen and, thus, were previously sensitized. In contrast to the recall immunized dogs shown in Figure 2A to 2D, this group of dogs did not receive transfusion of donor-specific blood 10 days before collection of PBMCs and start of culture. DCs from the respective DLA-identical donor were used as described above to stimulate mHA-specific CTLs. As shown in Figure 2G, there was no evidence of mHA-specific cytotoxicity of cultured cells.

mHA-Specific CTLs Were Not Isolated from Mixed Hematopoietic Chimeras

Mixed chimerism was established after a nonmyeloablative TBI-based conditioning regimen and a DLA-identical littermate marrow grafting [2,16]. PBMCs were obtained from 4 stable mixed hematopoietic chimeras 8 to 22 months after transplantation. Stimulatory DCs were obtained from the respective bone marrow donor and, separately, from DLA-nonidentical, unrelated dogs to stimulate the PBMCs ex vivo. After repeat in vitro stimulation with respective DLA-identical DCs, there was 0 to 8% (median, 2%) mHA-specific lysis at a 20:1 effector to target ratio (Figure 2H). The lack of response to DLA-identical DCs was not due to anergy or poor immune reconstitution. After 2 serial stimulations of PBMCs from mixed chimeras with DLA-nonidentical DCs, there was 28% to 45% allospecific lysis (data not shown).

mHA-Specific CTLs Lyse Bone Marrow Stroma and Fibroblasts

After 3 serial DC stimulations during the 28 days in culture, the mHA-specific CTLs from dogs previously sensitized and given recall immunization (treatment group A, Figure 2A to 2C) were assessed for ability to lyse stroma and fibroblasts. CTLs were added to DLA-identical littermate, autologous, and third party stroma in increasing amounts and assessed for cytotoxicity. There was 69% and 74% lysis of DLA-identical littermate donor stroma and fibroblast cells, respectively, with insignificant lysis of autologous or third party stroma or fibroblast cells (Figure 3).

Immunophenotype of mHA-Specific CTLs

After stimulation with DCs and 28 days in culture, the mHA-specific CTLs obtained from dogs previously sensitized and given recall immunization (treatment group A, Figure 2A to 2C) were 96% to 99% $CD3^+$, 70% to 85% $CD8^+$, and 16% to 31% $CD4^+$. Figure 4A and 4B show a representative histogram of

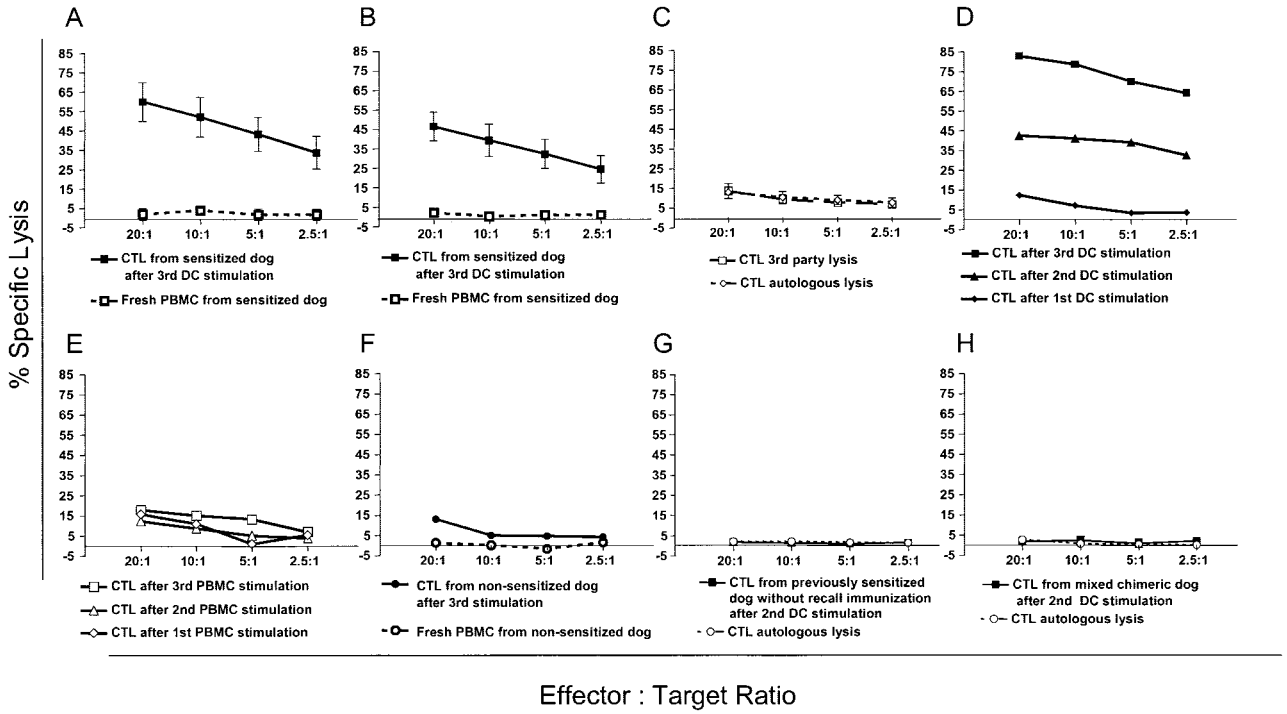

Figure 2. mHA-specific cytotoxic activity of cultured CTLs and freshly isolated PBMCs measured by ^{51}Cr release assay. Assay targets were DCs or con-A blasts from the respective DLA-identical stimulator dog with autologous con-A blasts and a panel of third party con-A blasts as controls. A to C depict the median specific lysis ± standard error of 6 separate experiments from all 4r dogs in treatment group A (previously sensitized dogs given recall immunization). Lysis of (A) respective DLA-identical donor DC targets, (B) respective DLA-identical donor con-A blasts, and (C) a panel of third party and autologous con-A blasts. (D) Representative example of enhanced mHA-specific activity after the first, second, and third DC stimulation. CTLs were cultured from PBMCs of sensitized dog E608 (treatment group A), with assessment of the percentage of specific lysis of the DLA-identical littermate E614 DC targets. (E) Representative example of poor ex vivo enrichment for specific lysis of mHA targets after serial stimulation with PBMCs. Cells from dog E608 (treatment group A) were serially stimulated with E614 PBMCs and assessed for lysis of E614 and E608 targets. (F) Minimal lysis of DLA-identical targets with cells obtained from nonsensitized dogs (treatment group C). (G) No evidence of enrichment of mHA-specific CTL lytic activity from T lymphocytes of dogs in treatment group B. Two recipients were previously sensitized to donor mHA but not given a recent donor-specific blood transfusion. After serial in vitro stimulation of PBMCs with donor DCs, there was no lysis of the respective DLA-identical targets. (H) Mixed chimeras (treatment group D) did not generate mHA-specific CTLs in vitro. Shown are results of median specific lysis ± standard error from 4 mixed chimeras serially stimulated with DLA-identical donor DCs.

the mHA-specific CTL immunophenotype (6 independent experiments).

Ex Vivo Expansion of mHA-Specific CTLs

Based on the starting number of PBMCs from dogs previously sensitized and given recall immunization (treatment group A) that were added to the allostimulatory DLA-identical DC culture on day 1, there was an initial decrease in the absolute number of viable cells after the first and second DC stimulation (Figure 4C). This was followed by a 2- to 6- fold expansion of alloreactive cells after the third DC stimulation. In contrast, there was a steady decrease in the number of viable T cells when DLA-identical PBMCs were used for stimulation of sensitized PBMCs or when PBMCs from nonsensitized dogs (treatment group C) were stimulated with DCs. In the latter 2 experimental conditions, the final cell contents in culture were 6% to 8% of the starting PBMCs. These results support the observation that DCs were superior to PBMCs as stimulators for expanding mHA-specific CTLs and that PBMCs from dogs previously sensitized and given recall immunization generated mHA-specific CTLs more efficiently.

DISCUSSION

In this study, mHA-specific CTL were reliably generated and expanded from PBMCs obtained from dogs previously sensitized in vivo and recently rechallenged through transfusion of donor-specific whole blood. Cytotoxicity was increased from <5% in the starting population of sensitized PBMCs to a median of 63% after 4 weeks in culture. DLA-identical littermate DCs were necessary for optimal ex vivo proliferation and expansion of mHA-specific CTLs. Under ex vivo culture conditions including 3 serial allogeneic DC stimulations, there was a progressive increase of mHA-specific CTL activity. The $CD34^+$-derived donor DCs used in these studies were at least 10-fold

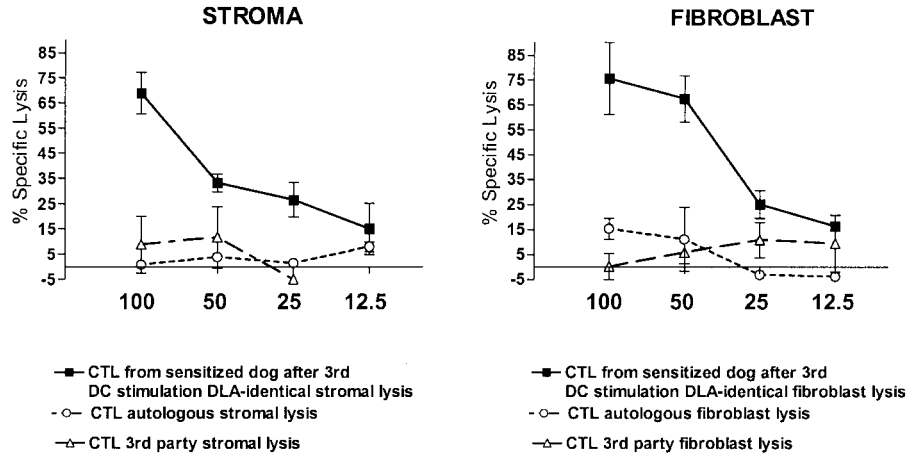

Figure 3. mHA-specific CTLs lysed bone marrow stroma and fibrolasts. CTLs from dogs sensitized and given recall immunization (treatment group A, Figure 3A to 3C) efficiently lysed bone marrow stroma and fibroblasts of the DLA-identical donor (solid line), but not of autologous or third party bone marrow stroma and fibroblasts (dashed line). The number of effector CTLs added per well $\times 10^3$ is indicated on the x-axis. Shown are results of median specific lysis ± standard error from the 3 dogs studied.

more efficient at expansion of allospecific CTL compared with the more cumbersome method of using DC-enriched PBMCs [10] based on the number of DCs needed to generate mHA-specific CTLs. Our results are consistent with the notion that DCs provide optimal presentation of mHAs ex vivo to stimulate CTL responses. Without prior in vivo sensitization of dogs, we were unable to generate mHA-specific CTLs using the current method of T-cell expansion. In addition, our results show that a donor-specific whole blood transfusion to boost the immune recall to mHAs was necessary to isolate mHA-specific CTLs from previously sensitized dogs. This may have been because of the low frequency of mHA-specific T cells circulating in the blood of nonsensitized dogs or animals sensitized in the more distant past.

Although hematopoietic cells were used to sensitize recipients in this study, the mHA-specific CTLs isolated from dogs were not tissue-specific. Both marrow stroma and fibroblasts were lysed in an mHA-specific manner. These results are consistent with previous findings in which stromal cells became targets of a GVH reaction in patients with poor marrow function after transplantation [23]. In addition, the results support the suggestion that immunogenic mHAs involved in marrow graft rejection can be shared among nonhematopoietic tissues.

We were unable to isolate mHA-specific CTLs from mixed chimeric dogs. Stable mixed hematopoietic chimerism after a nonmyeloablative preparative regimen in the dog reflects a state of donor-host immune tolerance. In this setting, skin and kidney grafts from the bone marrow donor are accepted indefinitely in the mixed chimera without further immunosuppression [24]. We have hypothesized that an active suppressor cell mechanism is responsible for maintaining the stability of mixed chimerism [5]. Our results suggest that alloreactive lymphocytes are not circulating in the mixed chimeras at high frequency despite the persistence of potentially allostimulatory mHAs. Whereas further studies are needed to reveal the mechanism of stable mixed chimerism, our results indicate that the tolerance mechanism in mixed chimeras blocks the emergence of alloreactive lymphocytes.

Although we report that mHA-specific CTLs were successfully isolated from dogs that had previously rejected DLA-identical littermate marrow grafts, the ex vivo expansion of mHA-specific CTLs is also feasible from dogs that have been sensitized by nonirradiated blood transfusions or serial skin grafts from DLA-identical littermates (unpublished results). These findings are potentially applicable to other experimental settings, such as the generation of mHA-specific T cells for adoptive immunotherapy. Ex vivo expansion of mHA-specific human CTLs is feasible, and preliminary clinical experience has been reported [25,26]. However, a large animal model of mHA-specific CTLs would be useful to accelerate clinical progress. Our results are particularly relevant to this aim.

There are more than 50 mHAs identified in the mouse and many that have been identified in humans, although several hundred minor antigens are postulated to exist in humans [27]. Although the mHAs responsible for the CTL expansion in this report were not identified, these results show a robust, general method for expanding mHA-specific CTLs in dogs that may be applied to any MHC-matched setting.

In summary, we describe the ex vivo isolation of mHA-specific CTLs from dogs previously sensitized and given recall immunization using DCs. Such mHA-specific CTLs could not be ex vivo expanded from nonsensitized dogs or mixed hematopoietic chimeras. The current results define a model system to permit the large-scale expansion of mHA-specific

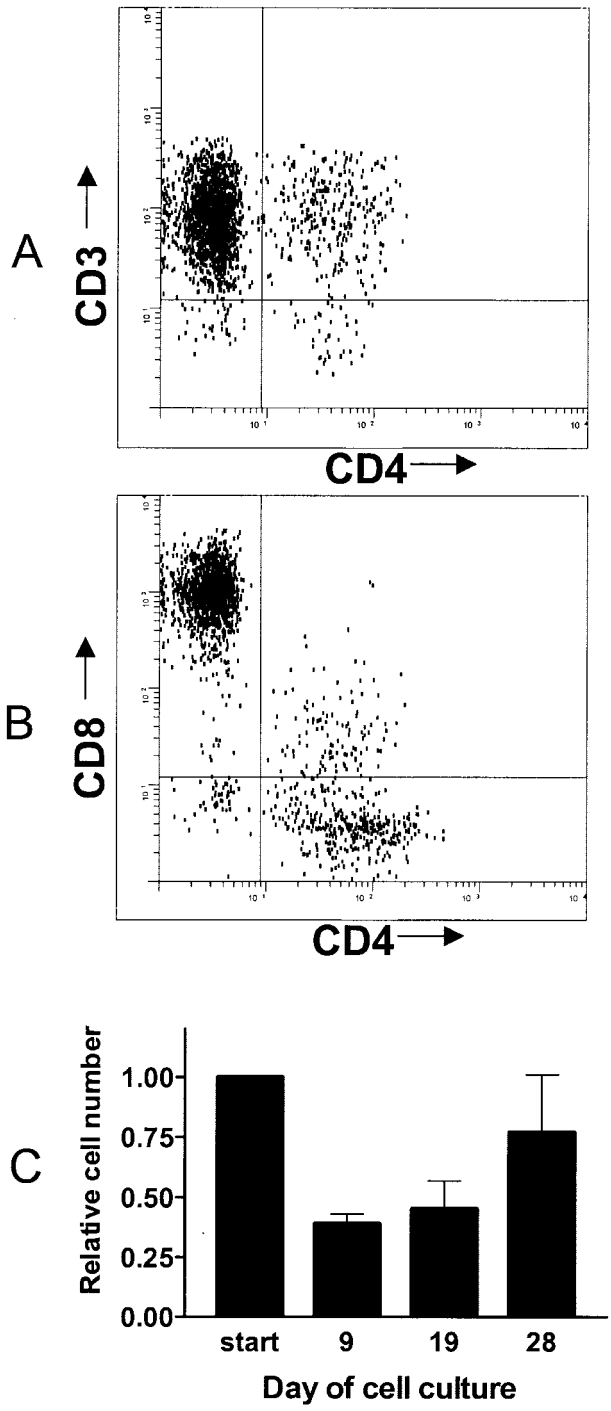

Figure 4. Immunophenotype and expansion of mHA-specific CTLs. (A and B) Representative example (n = 6) of the phenotype of mHA-specific CTLs on day 28 of culture (8 days after third DC stimulation) isolated from dogs in treatment group A. Cells were 97% $CD3^+$, 80% $CD8^+$, and 24% $CD4^+$. (C) Total viable cell number was measured using trypan blue dye exclusion at the beginning of culture and after first, second, and third DC stimulation (days 9, 19, and 28, respectively). Shown are results of 6 separate experiments that generated mHA-specific CTLs from 4 dogs previously sensitized and given recall immunization with the median cell number ± standard error normalized to the starting number of responder PBMCs. Despite the decrease in absolute cell number at the end of 28 days in culture, there was substantial enrichment of the mHA-specific cytotoxicity of the cultured cells compared with the starting population of freshly isolated PBMCs.

CTLs for in vivo studies in the dog model of HSCT. Because mHA-specific CTLs mediate graft rejection, isolation of these cells will facilitate development of new strategies to overcome resistance to engraftment in sensitized hosts. In addition, when the hematopoietic stem cell donor is sensitized to the recipient mHAs, the GVH activity of mHA-specific CTLs could be used to convert stable mixed chimerism to complete donor chimerism after nonmyeloablative HSCT. Conversion of mixed to complete donor hematopoietic chimerism is a surrogate for testing the GVL effect in a robust, preclinical large animal model. In vivo studies of mHA-specific CTLs would permit the testing of novel methods to control the risk of development of GVHD [28]. For example, we have previously shown that retroviral transduction with the herpes simplex virus thymidine kinase gene can confer ganciclovir sensitivity to allospecific CTLs [19]. In future studies, mHA-specific CTLs genetically modified with a suicide gene could be used to enhance the control and safety of the GVH effect after nonmyeloablative allogeneic HSCT.

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